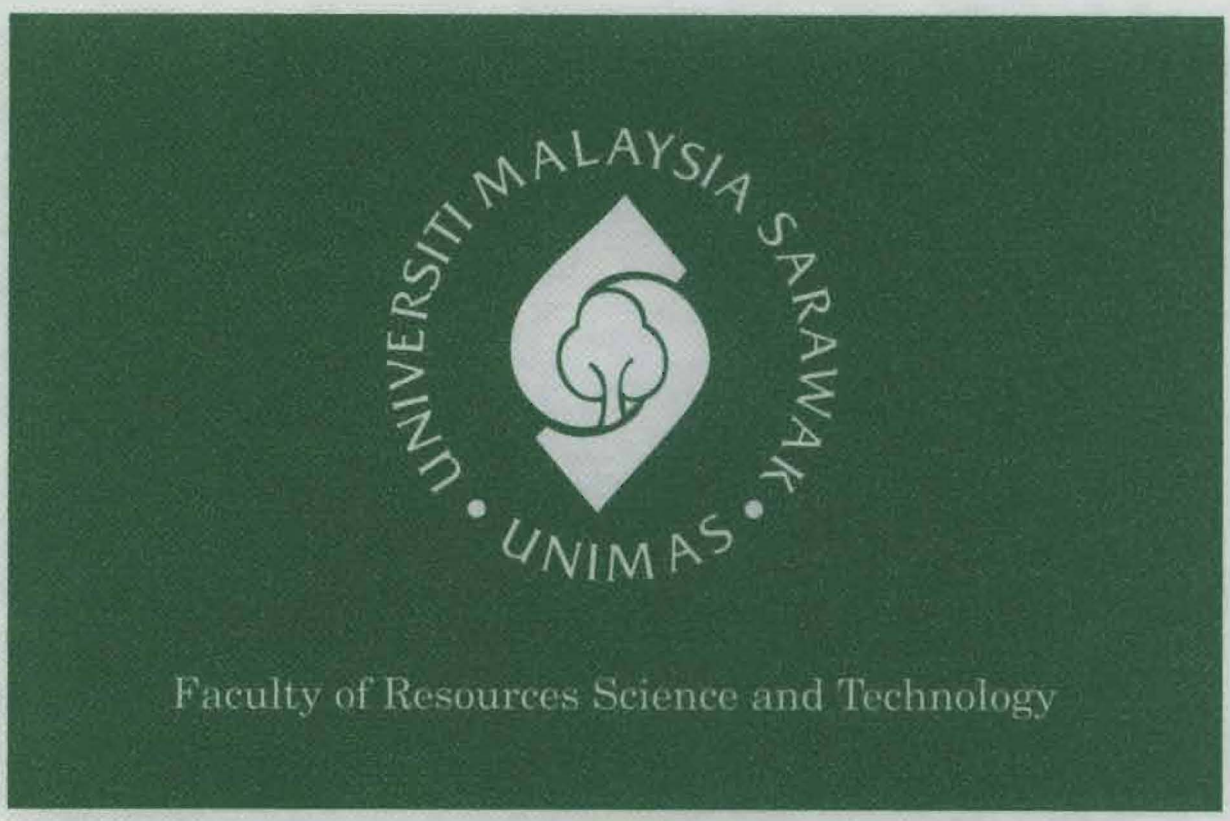

DETERMINATION OF OPTIMAL PARAMETERS IN THE PROTOCOL FOR TOTAL GENOMIC DNA ISOLATION FROM BLOOD SAMPLES OF THE JAMNAPARI GOATS

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Determination of Optimal Parameters in the Protocol for Total Genomic DNA Isolation from Blood Samples of the Jamnapari Goats

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List of Abbreviations

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
RNA	Ribonucleic acid
NaOAc	Sodium Acetate
OD	Optical Density
PC	Phenol-Chloroform
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulfate
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

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ABSTRACT

Jamnapari goat (*Capra hircus*) is an important economic goat breed originated from India with high milk-and-meat production. Genetic profiling is important for the verification and characterization of Jamnapari goat breed. A pre-requisite for successful implementation of these genetic resourcing methods is the ability to isolate a good amount of high quality total genomic DNA. Therefore, a reliable method producing good yield of high molecular weight DNA from blood samples is essential and required for downstream analysis. This study was conducted to determine the optimal parameters influencing the total genomic DNA extraction from blood samples of Jamnapari goats. The parameters studied included methods of extractions, incubation temperatures and time towards the quantity and quality of DNA extracted from blood. The DNA obtained from each parameter tested were amplifiable with *cytochrome b* primers giving a product size at a range of 300-400bp. The DNA sequence analysis showed that the products obtained were 99% identical to the sequences of Jamnapari goats. Analysis of variance showed that there were no significant differences between the phenol-chloroform method and salting-out method for DNA extractions. The optimal level of parameters for DNA extraction from whole blood sample of Jamnapari goat was by Salting-Out method with incubation temperature of sample digestion at 55°C and incubation time of 2 hours.

Key words: Jamnapari goat, DNA extraction, whole blood, phenol-chloroform, Salting-Out.

ABSTRAK

Kambing Jamnapari (Capra hircus) merupakan sejenis kambing yang berasal dari India. Kambing Jamnapari sangat bernilai dengan penghasilan susu dan daging yang tinggi. Profiling genetik amat penting dalam karekterisasi kambing. Untuk mendapatkan sumber genetik dengan berjaya, keupayaan dalam isolasi DNA dengan kuantiti yang banyak amat diperlukan. Dengan itu, satu kaedah yang dapat mengisolasikan kuantiti DNA yang tinggi dari sampel darah sangat penting dan amat diperlukan dalam analisis seterusnya. Kajian ini dijalankan untuk menentukan parameter yang optima dalam kaedah pengekstrakan DNA dari sampel darah kambing Jamnapari. DNA yang diekstrak dari setiap parameter yang diuji mempunyai kuantiti yang mencukupi untuk menjalankan amplifikasi dengan primer cytochrome b dan mendapatkan saiz produk dalam lingkungan 300-400bp. Analisis jujukan DNA menunjukkan DNA yang diekstrak mempunyai 99% kesamaan dengan kambing Jamnapari. Parameter yang dikaji termasuk kaedah pengekstrakan DNA yang digunakan, suhu dan masa inkubasi terhadap kualiti dan kuantiti DNA yang diekstrak dari darah. Varian analisis menunjukkan tiada perbezaan min antara kaedah pengekstrakan dengan fenol-klorofom dan salting-out. Paras optima bagi setiap parameter yang diuji dalam kaedah pengekstrakan DNA dari darah kambing Jamnapari ialah dengan menggunakan kaedah salting-out dengan suhu dan masa inkubasi pada 55°C untuk 2 jam.

Kata kunci: Kambing Jamnapari, Pengekstrakan DNA, darah, fenol-klorofom, salting-out

1.0 Introduction

Jamnapari goat (*Capra hircus*) is one of the most highly productive ruminants among all domesticated ruminants in tropical and subtropical conditions. This breed is a resourceful ruminant in producing several commercial products such as meat, milk, cheese and skin resulting in high demands of these goat breeds. (Anita *et al.*, 2007) The Jamnapari goat breed originated from India (Acharya, 1982) and is now bred in many parts of the tropical regions, including Malaysia.

Genetic profiling of Jamnapari goat (*Capra hircus*) is important for the verification and characterization of goat breed. It is also vital for identification of the genetic variability between different goats based on their DNA profile for the conservation of its valuable genetic resources. Genetics has a key role to play in helping animal farming to meet the world's growing demand for goats and to improve the performance of farmed goats. Goat genetic resources also help to determine the productivity of goat populations and their adaptability to environmental stresses, such as climate change and human development. A prerequisite step in creating a genetic profile and the foremost step involves in molecular biology is the ability to isolate a good amount of high quality genomic DNA from goats.

Goats genetic profiling requires a reliable source of biological material. The source of DNA can be from non-invasive sampling methods such as using hairs, feces or urine. These sources seem to be attractive since it allows genetic analysis without having to handle the livestock. However, this strategy usually results in a low quantity and poor quality of DNA which limits its potential (Adriane *et al.*, 2003). In contrast, sources that uses tissues such as blood or muscle tissues without critical damages to the animals, can exploit the full potential of DNA analyses including individual and sex determination,

relatedness estimates, pedigree reconstruction, determination of the effective population size and the level of genetic polymorphism within and between populations (Adriane *et al.*, 2003).

Nonetheless, the extraction of total genomic DNA from these sources is difficult. Blood contains high concentrations of proteins such as heme, electrolytes, enzyme inhibitors which can interfere with the quality of DNA for downstream analysis. Although there are many established protocols which can successfully isolate quality DNA such as phenol-chloroform method and salting-out method. However, these protocols differ widely concerning the initial sample volume, time and temperature of isolation, reagents required and the quantity and quality obtained. (Khosravinia *et al.*, 2007) Hence, this study aimed to improve previous protocol by studying the optimal parameters for DNA extraction from blood samples of the Jamnapari Goat (*Capra hircus*).

The objectives of this study were:

- To determine the optimal parameters in the protocol for the total genomic DNA isolation from blood samples of the Jamnapari goats (*Capra hircus*).
- To study the influence of DNA extraction methods, incubation temperatures, incubation time and sample storage duration towards the DNA extraction from blood samples.
- To evaluate the quantity and quality of DNA obtained so that an optimized protocol can be developed.

2.0 Literature Reviews

2.1 Jamnapari Goats (*Capra hircus*)

The India subcontinent has 20 well-characterized breeds of goats (*Capra hircus*), representing a wide spectrum of genetic variability which varies in their genetic potential for the production of milk, meat and fiber, disease resistance, heat tolerance and fecundity. (Joshi *et al.*, 2004) The genetic variability of indigenous breeds is important for conserving precious and irreplaceable genetic resources which may be applied to new productive demands.

The Jamnapari also known as Jamunapari or Etawah goat (*Capra hircus*) is named after an area beyond the river Jamuna where the breed was developed. Jamnapari goat is a dual purpose milk-and-meat goat breed which is mainly found in the undulated land of Chakarnagar between the ravines of the Jamuna (Yamuna) and Chambal rivers in the Etawah district of the Indian state of Uttar Pradesh. (Acharya, 1982). This breed is prolific and non-seasonal breeder. (Devendra, 1985) It has high economic value because they yield the highest amount of milk among all the Indian goat breeds. The milk yield is about 200kg per lactation and growth. (Acharya, 1982)

2.2 Genotyping Studies on Goats

The variability in genetic makeup of Jamnapari goats as well as the different management leads to variation in milk yield. According to Kumar *et al.*, (2006), genotyping studies on Jamnapari goats has been carried out to evaluate the genetic variability in exon-I of the α-lactalbumin milk gene using the non-radioactive PCR Single-strand Conformation Polymorphism (PCR-SSCP) method on molecular marker-assisted selection for improvement in milk yield and quality in Indian goat genetic resources.

Othman and Ahmed, (2007) also reported their genotyping studies on detection of genetic variants of kappa-casein (k-CN) which is the essential milk protein among four breeds of goats reared in Egypt using PCR Restriction Fragment Length Polymorphism (PCR-RFLP) technique. Identification of genotypes of Egyptian goat milk protein will enable the establishment of better breeding strategy for the production of higher quality milk.

Since goats are prolific ruminants, the knowledge on genetic variations within or among breeds is important for understanding and developing endogenous economic traits of breeds and for optimizing breeding strategies and regulating germplasm conservation. Studies were done by Anita *et al.*, (2007) on the genetic variations among six breeds of Indian goats using the Random Amplified Polymorphic DNA (RAPD) technology. The scarcity of polymorphic markers in the goat has been a major problem for construction of genetic linkage map. Hence, in a recent research by Ramamoorthi *et al.*, (2009) revealed the study of genetic characterization of Barbari goats using microsatellite markers for the conservation and genetic improvement of goats breed in India.

In Sarawak, Handalas Sdn Bhd. is a company engaged in goat breeding by using biotechnology. This company has ventured into the production of frozen semen for artificial insemination and embryo transfer for the improvement of goat breeding and sexing to determine the gender of livestock (Sharon, 2009). However, the feasibility of these extensive researches often limited by the difficulties encountered in isolating of quality DNA for a quick genetic profiling (Nelson *et al.*, 2008). Hence, developing simplified, rapid and efficient DNA extraction methods has become a major concern.

2.3 Mammalian Blood Components

Blood is a specialized bodily fluid that delivers essential substances such as nutrients and oxygen to the body's cells and transports waste products away from the cells. Mammalian blood contains inorganic components including the major electrolytes sodium, chloride, and potassium ions. Plasma contains soluble proteins, the most abundant of which is serum albumin. Other plasma proteins include immunoglobulins, fibrinogen, and clotting factors. The blood cells present in blood are mainly red blood cells which also called erythrocytes. There is only small concentration of nucleated white blood cells, including leukocytes and platelets present in mammalian blood. (Taylor *et al.*, 1997)

In molecular biology, blood sample has become one of the most commonly used specimens for genetic analysis. However, the high concentrations of heme group presence in the blood become contaminants in the total genomic DNA extraction. High concentration of proteins and other factors can interfere with the quality of DNA for the downstream analysis. The problem of extracting DNA from mammalian blood compared to other cell type is due to the high concentration of red blood cells. Red blood cells are anucleate and do not contain DNA. Unlike mammalian blood, the mature erythrocytes in avian and reptiles blood are nucleated and containing DNA. (Pendl, 2006) Hence preparation of genomic DNA from mammalian blood is more difficult and thus an optimized protocol is required.

2.4 Total Genomic DNA Extraction

There are many protocols for DNA extraction of high quality DNA that has been established and published over the last few decades. The protocols available vary in the initial sample volume, time and temperature of isolation, reagents used and the precision of method. (Khosravinia *et al.*, 2007)

The DNA extraction comprises of three major steps which include cell disruption, protein denaturation preliminary to the alcohol precipitation method. (William *et al.*, 2007) The conventional methods for DNA extraction that have been established are phenol-chloroform, isoproponal precipitation and salting out. (Godratollah & Adel, 2009) The demand for rapid and simplified protocols for DNA extraction has lead to the development of many commercial kits such as Arcturus, Invitrogen, Qiagen, Stratagene, Promega, and others. However, the yields of DNA are slightly lower compared to the conventional method. (Santella, 2006) Phenol-chloroform method is well-established in DNA extraction procedure even though it is known to be laborious and involves toxic reagents. (Carracedo, 2005) Nonetheless, this method yields good quality of DNA.

2.5 Parameters Influencing DNA Isolation

2.5.1 Lysis Buffer

Cell disruption is the preliminary step in DNA isolation protocol. Inaccessibility of cells to lysis forces will result in reduced yield and reproducibility. (Ginny *et al.*, 1999) The cells are disrupted by adding lysis buffer which will destabilizes the cell membrane and causes the breakdown of cellular structure so that the DNA will be released from the nucleus. Lysis buffer consists of Tris-HCl pH 8.5, ethylenediaminetetraacetic acid (EDTA) and Sodium Docecyl Sulfate (SDS) detergent. (Hogan *et al.*, 1994)

2.5.2 Proterinase K

Proteinase K is a type of endolytic enzyme which is isolated from the saprophytic fungus *Tritirachium album.* It has high stability in wide range of pH and temperature but denatures above 65°C. Proteinase K has high activity in the presence of SDS and EDTA whereby SDS can enhance the activity of Proteinase K by unfolding the proteins substrates causing them become more easily to be degraded. The incubation temperature of 55^0C is to increase the activity of the enzyme. (Kieleczawa, 2006)

2.5.3 Incubation Time and Temperature

The incubation of samples with lysis buffer and Proteinase K is a phase in which the cell lysis take place. The incubation time is highly depends on the cell type of the specimens used. The presence of cell wall or peptidoglycan require more time for lysis reaction. The optimization of this parameter is essential because if the incubation time is too long, it will lead to partial degradation of DNA. (Khosravinia *et al.*, 2007) On the other hand, short incubation time will causes incomplete lysis of the cell membrane and restrain the release of DNA from the nucleus resulting in significantly low yield of DNA obtained.

(Khosravinia *et al.*, 1997) The optimal incubation temperature for Proteinase K lies within the range of 55°C to 65°C. Temperature above 65°C will causes denaturation and inactivation of Proteinase K. Hence, incubation temperature is an important factor in affecting DNA isolation.

2.6 Agarose Gel Electrophoresis

A relatively rapid and easy method for assessing both the quantity and quality of extracted DNA is to visualize it on an agarose gel. Gel electrophoresis is a technique used in genetic analysis for the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein molecules that are different in size, charge or conformation by using an electric current applied to a gel matrix. (Robin, 1996)

Agarose is a polysaccharide extracted from seaweed algae. It is non-toxic compared to the possible presence of free acrylamide in polyacrylamide gel. The typically used concentration of agarose gel is in the range from 0.5 to 2%. In general, linear duplex molecules migrate through the gel matrix at a rate that is inversely proportional to the log of their molecular weight. Small DNA molecules move faster than large ones from cathode to anode. (William *et al.*, 2004)

2.7 UV Spectrophotometer Assay

According to William *et al.*, (2007), DNA absorbs light maximally at 260nm. This feature is utilized to estimate the amount of extracted DNA by measuring a range of wavelength from 220nm to 300nm. It can also be used to assess the amount of carbohydrates which has maximum absorbance at 230nm and protein at 280nm that may have co-extracted with the sample.

The DNA sample is placed in a quartz cuvette and scanning through spectrophotometer. According to Khosravinia *et al.*, (2006), the range of A_{260}/A_{280} ratio from 1.8 to 2.0 was considered pure. The A_{260}/A_{280} ratio of less than 1.7 indicates protein contaminations. The ratio higher than 2.0 might caused by RNA contaminations.

2.8 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) refers to an *in vitro* enzymatic DNA amplification strategy of producing millions of copies of DNA within a short period of time. A PCR technique was developed by Kary Mullis in 1985. Since then, PCR was applied extensively in the field of biological research and medical science. The application of PCR include the generation of hybridization probes for southern or northern blotting and DNA cloning which requires large amount of DNA. Since PCR allows the generation of large amount of DNA samples, it has been extensively modified to perform a wide array of genetic manipulations, diagnostic tests, and for many other uses. (Dennis *et al.*, 2006)

It involves a repetitive cycles of template denaturation, primer annealing and the extension of the annealed primer. The denaturation of DNA samples into ssDNA was done in high temperature of about 94°C. Next, the hybridization of the oligonucleotides to the targeted DNA template was aided by lowering the reaction temperature to about 54°C. The last step which is the extension occurs when the reaction temperature was increased to 72°C thereby speed up the process of synthesizing complementary DNA strand of targeted DNA sequence with the addition of dNTPs catalysed by *Taq* Polymerase in the 5'-3' direction forming double stranded copies of DNA. In the next cycle, newly synthesised DNA molecules will become the template DNA for subsequent reaction. (Dennis *et al.*, 2006)

3.0 Materials and Methods

3.1 Samples Collection and Preparation

The Jamnapari goat's specimens were collected from a goat farm located at Handalas Sdn. Bhd, Kuching. Blood samples were drawn from the jugular veins of 4 different Jamnapari goats using sterile syringes and needles. Blood samples were collected in sterile 3mL vacutainer collection tube containing 5.4mg of EDTA which functions as an anticoagulant. The blood samples were kept on ice to maintain low temperature in order to prevent hemolysis. Subsequently the blood samples were transported to the laboratory and stored at 4°C until the isolation of total genomic DNA. Ear tissues were collected by ear biopsy.

3.2 Total Genomic DNA Extractions

3.2.1 DNA Extraction from Whole Blood Samples

3.2.1.1 Phenol-Chloroform Method

The DNA from blood samples were extracted based on the protocol developed by the National Institutes of Health, (2004) with some modifications. Two hundred µL of whole blood sample was added with 500µL of Red Blood Cells lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM Na_2EDTA, pH7.4). The mixtures were gently shaken and incubated on ice for 30 minutes. Subsequently, the mixtures were centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended with 250µL of Red Blood Cells lysis buffer. The mixtures were centrifuged again at 10000 rpm for 10 minutes at 4°C.

The supernatant was removed and the pellet was resuspended with 200μL of SE buffer at pH8 which consisted of 75mM of NaCl and 25mM of Na_2EDTA. The mixtures were centrifuged at 10000 rpm for 10 minutes at 4°C. Then, the supernatant were removed and the pellet was resuspended with 200μL of SE buffer, 25μL of Proteinase K (10mg/mL) and 100μL of 10% SDS. The mixtures were then incubated at different incubation temperatures and incubation times.

Temperature (°C)	Time (hrs)
37	2
55	2
55	4

After incubation, the mixtures were added with 100μL SE buffer and equal volumes of phenol-chloroform were added to the mixtures. The mixtures were then centrifuged at 10000 rpm for 5 minutes at 10°C. The supernatant were transferred into fresh tubes and were added with 1/10 volume of 3M sodium acetate and 1 volume of isopropanol. The DNA was precipitated and the captured with a wide bore tip. The DNA was washed with 70% ethanol and finally resuspended in 40μL of nuclease-free water.

3.2.1.2 Salting-out Method

The salting-out method for DNA extraction from whole blood is similar to the phenol-chloroform. This method eliminated the phenol extraction steps. However, 6M saturated NaCl was used in replacement for DNA extractions.

3.2.2 DNA Extraction from Tissues

DNA was achieved from tissue sample of Jamnapari goats by the standard phenol-chloroform method described by Hogan *et al.*, (1994).

Starting sample volume of about 25mg was transferred into sterile 1.5mL microcentrifuge tubes. Lysis buffer of 500 µL and 25 µL of Proteinase K (10 mg/mL) were added into the tube. The lysis buffer consisted of 50 mM Tris buffer (pH8), 0.1M EDTA and 0.5% SDS (Sodium Dodecyl Sulfate). The mixture was incubated for one hour at 55°C in a waterbath.

The extraction of DNA was done by adding an equal volume of phenol: chloroform (1:1). The mixture was gently vortexed and was centrifuged at maximum speed (14000rpm) for 10 minutes until the mixture was separated into 3 phases. An aqueous phase on the top, a whitish interface, and an organic solvent phase at the bottom were observed. This step was performed in a laminar flow hood to avoid contaminations due to the toxicity of phenol.

The aqueous phase containing genomic DNA was transferred into a fresh 1.5 mL microcentrifuge tube. Precipitation of DNA was carried out by adding 1/10 volume of 3M NaOAc (pH 6.0) followed by 1 volume of absolute ethanol. The solution was mix gently and the DNA was pelleted by centrifugation at maximum speed (14000rpm) for 5 minutes. The DNA pellet was then washed with 1 mL of 70% ethanol, and the pellet was air-dried at room temperature until there is no droplet. The dried pellet was resuspended in 40µL of nuclease-free water.

3.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed for assessing both the quantity and quality of extracted DNA. One % (w/v) agaorse gel was prepared by diluting 0.4g of agarose in 40mL of 1 x TAE buffer in a microwave for 1 minute until it became hyaline without any gel particles. Then, 0.8μL of ethidium bromide was added to the mixture and poured to a gel tray. The comb is inserted as soon as the gel was poured into the casting tray in the electrophoresis chamber before the gel became solidified for wells formation. When the gel was solidified, the comb was removed. The casting tray was placed in the electrophoresis chamber with the wells on the cathode because the DNA are negatively charged molecules and it will move to positive electrode during the gel electrophoresis. The casting tray was covered with 1 x TAE buffer.

To perform gel electrophoresis, 1μL of 6X Loading dye will be added into 2μL of ladder (1Kb) and 5 μL of each DNA sample. The mixtures were mixed well and were loaded into the wells accordingly with ladder loaded in the first slot. Then, power supply will be connected and switched on with a constant voltage of 100V for 25 minutes to carry out the gel electrophoresis. The gel was then being visualized using the Gel Documentation.

3.4 DNA Quantification by UV Spectrophotometer Assay

The quality and quantity of DNA were accessed by scanning with a spectrophotometer. The samples were diluted with sterile distilled water at the ratio of 1: 100. For each sample, 10µL of extracted DNA is diluted with 990µL of sterile distilled water in a cuvette. The measurement of DNA purity will be determined by assessing the amount of ultraviolet irradiation absorbed by the bases in DNA make up. (Khosravinia *et al.*, 2007)

The DNA concentrations and purity of DNA were then determined with the following formula:

$$\text{DNA concentration } (\mu g/\mu l) = (A_{260} - A_{320}) \times 50\ (\mu g/\mu l) \times \text{Dilution Factor}$$

$$\text{Purity of DNA} = A_{260}/\ A_{280}$$

3.5 Polymerase Chain Reaction (PCR)

The amplification of DNA using PCR was performed in the Peltier Thermal Cycler MJ Research PTC-200. The PCR was carried out in a 12.5µL reaction volume for the amplification of the *cytochrome* b gene region. The reaction mixture consisted of GoTaq®Flexi Buffer, 25mM $MgCl_2$ solution, 10mM dNTPs, 1 unit *Taq* DNA polymerase and 25pmol/µL of each primer (*Cyt*b 1, 5' CCA TCC AAC ATC TCA GCA TGA TGA AA 3'; *Cyt*b 2, 5'GCC CCT CAG AAT GAT ATT TGT CCT CA 3'). (Ong *et al.*, 2007) The parameters for the amplifications were initiated with a hot start of 94°C for 5 minutes, 30 cycles of 30 seconds at 94°C, 56°C and 72°C consecutively, followed by a 10 minutes of final extension at 72°C. The PCR products were verified by 1.5% agarose gel electrophoresis containing ethidium bromide. The expected product size is 358bp.

3.6 Gel Purification for DNA Sequencing

Gel purification was done by using the Wizard® SV Gel and PCR Clean-Up System. Gel slice was prepared by loading 50μL of each PCR products on 1.5% agarose gel and run gel electrophoresis. The 1.5mL microcentrifuge tubes for each DNA fragments to be isolated were weighed and the weights were recorded. After gel electrophoresis, the gel was visualized using UV transilluminator. The DNA fragments of interested were excised in a minimal volume of agarose gel using a clean scalpel blade. The gel slices were transferred to the weighed microcentrifuge tubes and the weights were recorded again. The weight of the gel slices were obtained by subtracting the weight of empty tube from total weight. Membrane Binding Solution was added at a ratio of 10μL of solution per 10mg of agarose gel slice. The mixtures were vortexed and incubated at 55°C for 5 minutes or until the gel slices were completely dissolved.

Gel purification is followed by the recovery of DNA from the gel slices. The SV Minicolumn was placed in a Collection Tube for each dissolved gel slice. The dissolved gel mixtures were transferred to each SV Minicolumn assembly and incubated at room temperature for 1 minute. The SV Minicolumn assembly were centrifuged at 14000rpm for 1 minute. The liquid in the Collection Tube was discarded and the SV Minicolumn were returned to each Collection Tube. The columns were washed with 700μL Membrane Wash Solution diluted with 95% ethanol. The SV Minicolumn assembly were centrifuged at 14000rpm for 1 minute. The liquid in the Collection Tubes were discarded and the SV Minicolumn were placed back and washed again with 500μL of Membrane Wash Solution. The assembly were centrifuged at 14000rpm for 5 minutes. The SV Minicolumn assembly were removed from the centrifuge gently without wet the bottom of the column with the flowthrough. The Collection Tubes were emptied and the columns were